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Award Number: DAMD17-01-1-0545

TITLE: PIAS1 and PIAS3 Protein Interaction with HsRad51 and HeRad52 May Downregulate Recombination Repair in Human Mammary Epithelial Cells

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REPORT DATE: August 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Final (01 Aug 01 - 31 Jul 02)

4. TITLE AND SUBTITLE
PIAS1 and PIAS3 Protein Interaction with HsRad51 and HeRad52 May Downregulate Recombination Repair in Human Mammary Epithelial Cells

5. FUNDING NUMBERS
DAMD17-01-1-0545

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Characterization of *BRCA1* and *BRCA2* has strongly implicated homologous recombinational repair (HRR) as a pathway important in breast cancer. Rad51 and Rad52 are two additional proteins important for HRR, and we are characterizing some proteins that interact with them. Using the yeast two-hybrid system, we had shown that the human proteins PIAS1 and PIAS3 (protein inhibitors of activated STATs) specifically interact with Rad51 and Rad52. Recently, PIAS1 has been shown by others to interact with p53 and to be involved in its sumoylation. Sumoylation is related to ubiquitination, but does not appear to tag a protein for degradation.

During the last year we confirmed the interaction between PIAS1 and hRad51 using purified hRad51 and PIAS1-GST fusion proteins. In addition, the regions of PIAS1 that interact with Rad51, Rad52 and P53 were mapped and shown to completely overlap. This region is the acidic domain of PIAS1 (PIAS1-AD). The interacting region has been further narrowed to 21 amino acids, and we are currently mutating individual residues to determine which mediate these interactions. We have also shown that both PIAS1 and PIAS1-AD interact strongly with WRN and weakly with BLM, proteins defective in Werner and Bloom syndromes associated with increased cancers.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, PIAS1, PIAS3, HsRad51, HeRad52	8
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

PIAS1 and PIAS3 (PIAS1/3) were identified as inhibitors of the STAT1/3 transcriptional activators. PIAS family members (PIAS1/3, PIASxα/β and PIASy) also interact with other transcriptional activators, including the estrogen receptor (ER) and P53, proteins implicated in breast cancer. We have previously shown that PIAS1/3 also interact with Rad51 and Rad52, human proteins involved in homologous recombinational repair (HRR). HRR is a DNA repair pathway that may be defective in many breast cancers (Thompson and Schild, 2001). The purpose of this DOD Breast Cancer Concept Award was to better characterize general aspects of the interactions between PIAS1/3 proteins with the human Rad51 and Rad52 proteins. As outlined below, we have made significant progress towards this goal. Another goal was to determine if the PIAS1/3 proteins might act as down-regulators of HRR by binding to and thus inactivating either the DNA-binding or self-interacting domains of the Rad51 and Rad52 proteins. Although we have not answered this question yet, during the single year of this grant we have made considerable progress in this direction (discussed below).

BODY

We have made a number of interesting observations during the last year (described in greater detail below). Using the yeast two-hybrid system and *in vitro* interaction studies, we have mapped the region of PIAS1 that interacts with Rad51, Rad52 and P53. This 22 amino acid region is called the acidic domain (PIAS1-AD). We have also shown that both PIAS1 and PIAS1-AD interact with the proteins defective in Werner and Bloom syndromes, associated with greatly increased cancer rates. Other laboratories have recently shown that the PIAS1/3 proteins are SUMO-1 (small ubiquitin-like modifier) E3 ligases (Kahyo *et al.*, 2001; Schmidt and Muller, 2002), involved in the recognition and sumoylation of specific proteins, including P53. Sumoylation can protect proteins from degradation or can affect their subcellular localization. The function of the PIAS1/3 interactions with transcriptional activators and with DNA repair proteins may be primarily sumoylation, or may involve more direct regulation (*e.g.* PIAS1/3 binding may block oligomerization or DNA-binding). By studying the interacting acidic domain of PIAS1, we hope to eventually develop peptides for analyzing recombinational repair and transcriptional activation. These peptides might also be useful for breast cancer treatments if they interact with ER.

Using the yeast two-hybrid system, we have mapped the region of PIAS1 that interacts with Rad51 and Rad52 (Fig. 1A).

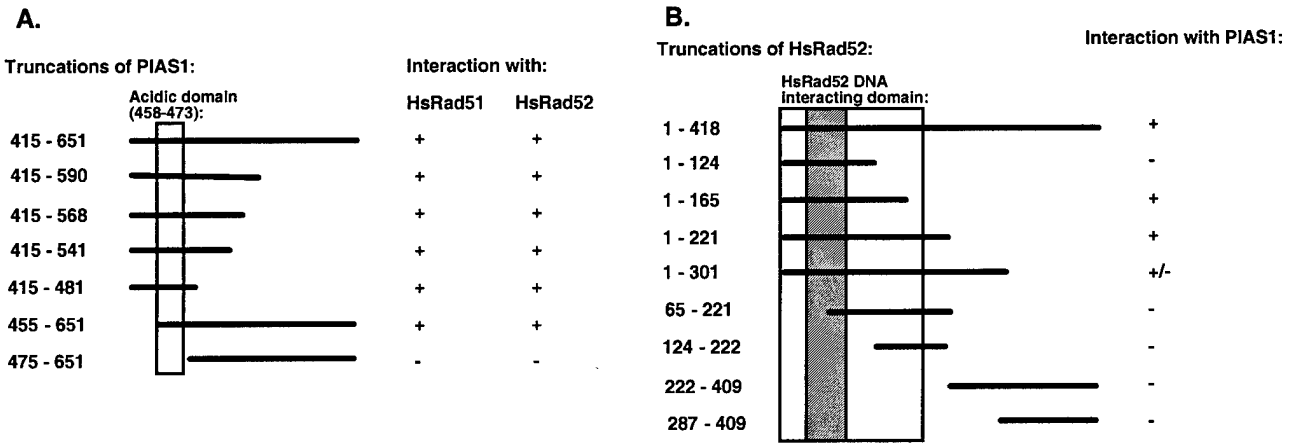

Fig. 1. Mapping the regions of PIAS1 and of Rad52 involved in interactions. A. PIAS1 truncations were constructed in pACT, a GAL4 transactivation domain vector, and human Rad51 and Rad52 were cloned into pGBT9, a GAL4 DNA-binding domain vector. β-galactosidase activity was assayed as in Fig. 2. **B.** HsRad52 truncations were similarly tested for interaction with PIAS1 (discussed below).

The yeast two hybrid system involves cloning two proteins or protein fragments into two different vectors to produce fusion proteins with different domains of the yeast GAL4 protein. These plasmids are then both transformed (equivalent to transfection in mammalian cells) into a yeast haploid strain with genetic markers that are used to assay (by expression of β-galactosidase or by growth on media lacking histidine or adenine) for interaction between the two proteins. This method was used to characterize interactions between PIAS1-AD and various partners (Figs. 1 - 3), and was used to map the domain of HsRad52 that interacts with PIAS1 (Fig. 1B).

The amino acid region of PIAS1 that interacts with Rad51 and Rad52 is very acidic, and is called the acidic domain (PIAS1-AD, aa 456 to 477: KVEVIDLTIDSSSDEEEEEPSA). Using this information, we have constructed a two-hybrid vector that only contains this 22 amino acid region and have confirmed that this region is sufficient for interaction with Rad51 and Rad52. We have also shown that this region is sufficient for interaction with P53 (Fig. 2).

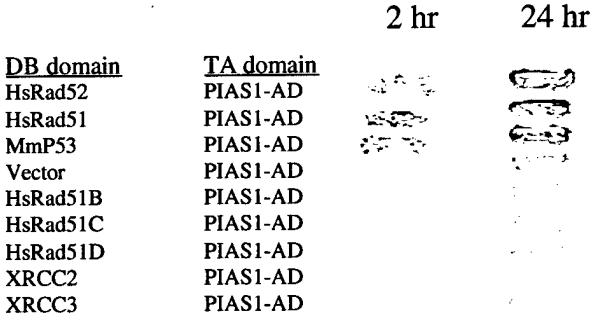

Fig. 2. Interaction of Rad51, Rad52 and P53 with PIAS1-AD (22 aa region) in yeast two-hybrid system. DB domain: DNA-binding domain plasmid and TA domain: transactivation domain plasmid. Yeast cells containing both plasmids were transferred to nitrocellulose, placed in liquid nitrogen to permeabilize cells and placed on X-gal to monitor β-galactosidase activity (blue color). Note: The vector control shown is pGBKT7, the vector used for construction of the MmP53 construct, and pGBT9, the vector used for all of the other constructs has a lower background (not shown).

The interactions observed are very specific, since neither PIAS1/3 nor PIAS1-AD interacts with any of the human Rad51 paralogs (Fig. 2, and data not shown), proteins that share sequence homology with Rad51. PIAS1-AD does interact with the Rad51 protein from yeast (data not shown), which is not surprising since the yeast and human proteins are highly conserved. We have also shown that changing the first I residue in PIAS1-AD to A (I460A) blocks the observed interactions, and even switching the I and D residues (I460D, D461I) blocks the observed interactions (data not shown).

Since both the Werner Syndrome protein (WRN) and the Bloom syndrome protein (BLM) are sumoylated (Kawabe *et al.*, 2000; Suzuki *et al.* 2001), we tested if either interacts with PIAS1 in the yeast two-hybrid system. We have shown that both PIAS1 and PIAS1-AD interact with WRN and weakly with BLM (Fig. 3 and data not shown), syndromes associated with a greatly increased rate of various cancers.

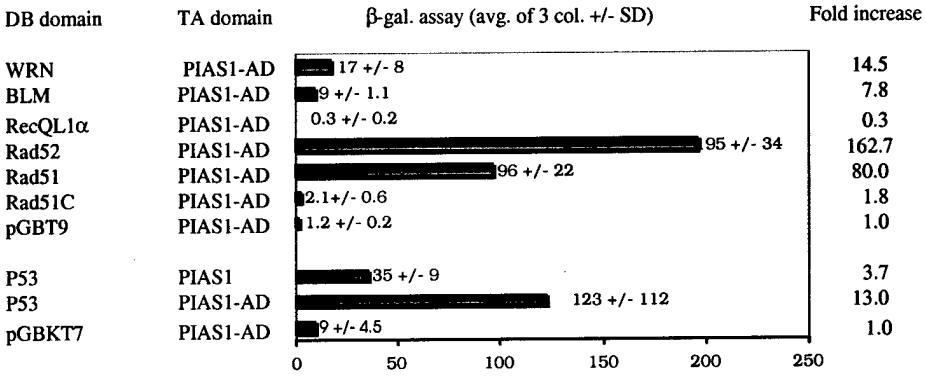

Fig. 3. Interactions of PIAS1-AD with several proteins, including WRN and BLM. The β-galactosidase activity was determined by chemiluminescence (units are relative) for three independently transformed colonies. Quantification confirms many of the results in Fig. 2, and shows a moderate interaction between WRN and BLM

with PIAS1-AD. The pGBT9 vector was used to construct the first six DB domain constructs and pGBKT7 was used for the P53 construct. None of the DB domain constructs gave significant background with just the vector in the TA domain (data not shown). The P53 construct lacks the first 71 aa that act as a transactivator on their own.

In vitro interaction studies using GST fusions and purified proteins are being used in some cases to confirm our two-hybrid results. Two-hybrid experiments showing an interaction between the acidic domain of PIAS1 with human Rad51 have already been confirmed using this GST fusion technique (Fig. 4).

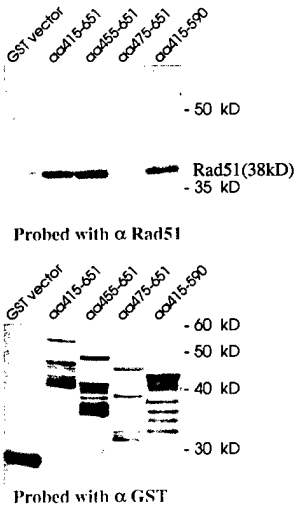

Fig. 4. Use of PIAS1-GST fusions to confirm the interaction with purified human Rad51 protein. Truncations of PIAS1 were fused to GST and the fusion proteins isolated after expression in *E. coli*. These fusions were incubated with purifed Rad51 protein, kindly supplied by Dr. Patrick Sung. The Rad51 protein co-eluted with PIAS1 only when the PIAS1 truncation included the acidic domain (aa 458-473), as can be seen by Western analysis after complexes are disassociated by denaturation. In the bottom panel, the top band in each lane is the correct size for the PIAS1-GST fusion protein. Degradation of fusion proteins are frequently seen in this type of experiment.

Isolation and characterization of point mutations in PIAS1-AD. We have already started to isolate point mutations in PIAS1-AD (discussed above). These mutations were made by substituting duplexed oligonucleotides into the two-hybrid plasmid encoding the PIAS1-AD. To simplify this substitution, we have incorporated a unique restriction site in the middle of the region encoding the 22 amino acids. To make a mutation we substitute only half of this region, making the oligos shorter, cheaper and less prone to secondary, unwanted mutations. Once potential mutations have been substituted, the PIAS1-AD region is sequenced to ensure that the expected mutation is present. A major goal is to isolate mutations that block some, but not all interactions.

Mapping interacting regions on proteins that bind the PIAS1-AD. The PIAS-interacting domain on human Rad52 has been partially mapped, using the two-hybrid system. Dr. Z. Shen has many Rad52 truncation mutations in two-hybrid plasmids (Shen *et al.*, 1996) and kindly supplied us with these. These have allowed us to determine that the region of Rad52 that binds PIAS1 overlaps the DNA-binding domain of Rad52 (Fig. 1B). The DNA-interacting domain has been localized to the region in the large box in Fig. 1B. Data cited as unpublished indicates that it has been mapped to the smaller hatched box, but since this data is unpublished, it is hard to evaluate. Since the self-interaction domain (for oligomerization) also maps near this region, only further studies will determine which region PIAS1 interacts with.

KEY RESEARCH ACCOMPLISHMENTS:

- The region of PIAS1 that interacts with Rad51, Rad52 and P53 has been mapped to the 22 amino acid region known as the acidic domain (PIAS1-AD).
- The interaction of purified Rad51 with PIAS1 and with PIAS1-AD has been confirmed *in vitro* by co-precipitation with PIAS1-GST fusion proteins.

- PIAS1 has been shown to interact strongly in the yeast two-hybrid system with the Werner syndrome protein (WRN) and weakly with the Bloom syndrome protein (BLM), and the interacting regions on PIAS1 have both been mapped to PIAS1-AD.

- The region of Rad52 that interacts with PIAS1 has been mapped to a region that contains both the DNA binding and self-interacting domains.

- The isolation and characterization of point mutations in PIAS1-AD has begun and two mutations have already been characterized.

REPORTABLE OUTCOMES

Two invited talks were presented in which about half of the material was based on the work funded by this grant. One was at the Radiation Research Society Annual Meeting (April 20-24, 2002 in Reno, Nevada) and the other at the Environmental Mutagen Society (EMS) Annual Meeting (April 27–May2, 2002 in Anchorage, Alaska).

Applications has been submitted for a DOD BCRP Idea Grant and for a DOD PCRP Idea Grant and these proposals are based on the work funded by this BCRP Concept Grant.

CONCLUSIONS

The acidic domain of PIAS1 is an important region for the interaction with a number of proteins involved in DNA repair, including Rad51 and Rad52. PIAS1 also interacts through this domain with the WRN protein and possibly with the BLM protein as well. The region of Rad52 involved in the interaction with PIAS1 overlaps its DNA-binding and self-interaction domains. The acidic domain of the PIAS proteins may be a useful target for disrupting these interactions, and therefore may have medical implications.

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APPENDICES – None.